Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03088.x

Preferential recognition of the phosphorylated major linear B-cell epitope of La/SSB 349–368aa by anti-La/SSB autoantibodies from patients with systemic autoimmune diseases

A. G. Terzoglou, J. G. Routsias, S. Avrameas, H. M. Moutsopoulos and A. G. Tzioufas
Department of Pathophysiology, School of Medicine, University of Athens, Athens, Greece

Summary

Sera from patients with primary Sjögren Syndrome (pSS) or Systemic Lupus Erythematosus (SLE) often contain autoantibodies directed against La/SSB. The sequence 349–368aa represents the major B-cell epitope of La/SSB, also it contains, at position 366, a serine aminoacid residue which constitutes the main phosphorylation site of the protein. In this study we investigated the differential recognition of the 349–368aa epitope and its phosphorylated form by antibodies found in sera from patients with systemic autoimmune diseases. Peptides corresponding to the sequence of the unphosphorylated (pep349–368aa) and the phosphorylated form (pep349–368aaPh) of the La/SSB epitope 349–368aa, as well as to a truncated form spanning the sequence 349–364aa and lacking the phosphorylation site (pep349–364aa), were synthesized. Sera from 53 patients with pSS and SLE with anti-La/SSB specificity, 30 patients with pSS and SLE without anti-La/SSB antibodies, 25 patients with rheumatoid arthritis and 32 healthy individuals were investigated by ELISA experiments. Autoantibodies to pep349–368aaPh were detected in sera of anti-La/SSB positive patients with a higher prevalence compared to the pep349–368aa (66% *versus* 45%). Pep349–368aaPh inhibited the antibody binding almost completely (92%), while pep349–368aa inhibited the binding only partially (45%). Anti-La/SSB antibodies presented a higher relative avidity for the phosphorylated than the unphosphorylated peptide. Immunoadsorbent experiments using the truncated peptide pep349–364aa indicated that the flowthrough showed a selective specificity for pep349–368aaPh, while the eluted antibodies reacted with both peptide analogues of the La/SSB epitope. These data suggest that sera from pSS and SLE patients with anti-La/SSB reactivity possess autoantibodies that bind more frequently and with a higher avidity to the phosphorylated major B-cell epitope of the molecule.

Keywords: B-cell epitopes, autoantibodies, La/ SSB, Sjogren's syndrome, post-translational modifications

Accepted for publication 8 March 2006
Correspondence: Dr A. G. Tzioufas, Department of Pathophysiology, School of Medicine, 75 M. Asias st, 11527 Athens, Greece.
E-mail: agtzi@med.uoa.gr

Introduction

Patients with primary Sjogren's syndrome (pSS) and Systemic Lupus Erythematosus (SLE) often have autoantibodies directed against the Ro/LaRNP ribonucleoprotein complex. This consists of small cytoplasmic RNA molecules (hYRNA) in noncovalent binding with different proteins, including La/SSB, Ro52 and Ro60 kD [1,2]. Human La/SSB is a phosphoprotein consisting of 408 aminoacids, with a molecular weight ranging from 48 to 51 kD. It contains three RNA recognition motifs (RRMs), aminoacid regions that are involved in binding to RNA molecules. A nuclear localization signal (NLS), which is located at the carboxy-terminal domain (CTD) of the molecule, allows the protein to move through the nuclear membrane. The major physiological role of La/SSB in the cell is to bind newly synthesized RNA molecules, transcribed by RNA polymerase III, such as tRNA and hYRNA, protecting them from the action of 3′-exonucleases and promoting their maturation [3,4].

La/SSB is phosphorylated primarily at the serine 366 aminoacid residue (Ser366) [5]. The phosphorylation of this residue is catalysed by the Casein Kinase II (CKII) enzyme. The

phosphorylation of La/SSB has been shown to affect the ability of the protein to bind the nascent RNA transcripts. More specifically, studies have shown that phosphorylation of the Ser366 decreases the ability of La/SSB to bind and subsequently protect the 5′-end of the RNA molecules [3].

Previous experiments from our laboratory defined the linear B-cell epitopes of La/SSB protein and showed that the aminoacid region 349–368aa represented the major linear B-cell epitope of the protein. It was found, firstly, that this epitope is recognized by autoantibodies in the sera of pSS patients with the highest specificity and sensitivity, secondly, that it is the end result of epitope spreading in rabbits immunized with the other La/SSB epitopes and finally, that autoantibodies against the 349–364aa are highly correlated with the HLA-DQA1*0501, the genotype associated with pSS [6–8]. This epitope also contains the aminoacid Ser366 that is primarily phosphorylated by the CKII enzyme.

In this study, we investigated whether the addition of a phosphate group to the Ser366 residue of the major B-cell epitope 349–368aa of La/SSB protein might influence its antigenicity. Compared to the unphosphorylated peptide analogue, it was found that the phosphorylated peptide was recognized more frequently and with a higher relative avidity by autoantibodies derived from sera of patients with pSS and SLE.

Materials and methods

Human sera

Sera were obtained from 53 patients with either SLE ($n = 13$) [9] or pSS ($n = 40$) [10], with anti-La/SSB autoantibodies detected by counter-immunoelectrophoresis (CIE) and/or immunoblot. Twenty-five sera from patients with rheumatoid arthritis (RA) [11], without anti-La/SSB antibodies, were used as disease controls, 30 sera from pSS and SLE patients, without anti-La/SSB and Ro/SSA antibodies, were used as autoantibody controls and 32 sera from healthy individuals were used as negative controls. All sera had been taken for diagnostic purposes with the full consent of the patients that part of the serum will be used for research purposes. Ethical approval for the study was obtained from the Scientific Committee of Laiko Hospital.

Peptide synthesis

Two synthetic peptides corresponding to the sequence of the 349–368aa epitope of human La/SSB were synthesized, utilizing the solid phase peptide synthesis (Biosynthesis Inc., Lewisville, TX, USA). The first peptide corresponded to the sequence of La/SSB NH_2–^{349}GSGKGKVQFQGKKTKFA SDD^{368}-CONH (pep349–368aa) and the second to its phosphorylated form (pep349–368aaPh), which has a phosphate group added to the Ser366. In addition, a peptide corresponding to the truncated form of the 349–368aa epitope NH_2–^{349}GSGKGKVQFQGKKTKF^{364}-CONH (pep349–364aa) was synthesized. This peptide is the shortest form of the epitope 349–368aa of La/SSB recognized by antibodies found in the sera of patients with pSS, and lacks the phosphorylation site Ser366 [12]. An irrelevant peptide IASRYDQL, corresponding to the sequence 250–257aa of Leismania glycoprotein gp63, was also constructed to be used as a control peptide (Ctrl-pep). All peptides were purified by High Performance Liquid Chromatography (HPLC). Peptide purity and the correct orientation of the phosphorylation were evaluated by Mass Spectra (MS) analysis.

Purification of human anti-349–364aa antibodies

Total IgG antibodies from the sera of three patients, containing autoantibodies to both the unphosphorylated and the phosphorylated forms of the La/SSB epitope, were purified by affinity chromatography using a protein-A Sepharose 4B column. IgG fractions were concentrated and dialysed against phosphate buffer saline (PBS). A specific immunoaffinity column of cyanogen bromide (CNBr) activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) was generated by standard methods, using 20 mg of the synthetic pep349–364aa. Anti-349–364aa IgG antibodies were purified from the three total IgG fractions. Pep349–364aa coupled beads were preincubated overnight at +4 °C, with approximately 100 mg of total IgG. Subsequently the beads were packed in a column washed with PBS and the flowthrough (IgG not bound to the beads) collected. Bound anti-349–364aa IgG antibodies were eluted with 0·1 M HCl-Gly, pH = 2·7. The flowthrough from the first purification was applied to the column once more to ensure that the majority of the anti-349–364aa antibodies were isolated. The two flowthroughs, as well as the eluents from the two consecutive experiments were mixed together, dialysed overnight against PBS (pH = 7·4), and protein concentration was measured using the Bradford assay.

Anti-peptide ELISA assays

All sera, as well as the flowthroughs and the eluents from the affinity column, were tested by specific anti-peptide ELISA experiments developed for the detection of antibodies against the peptide analogues of the 349–368aa La/SSB epitope. Briefly, 96-well polysterene plates with a hydrophilic surface (Multisorp^{TM}, NUNC, Denmark) were coated with either pep349–368aa or pep349–368aaPh, diluted in carbonate-bicarbonate buffer, pH = 8·6, at a concentration of 5 μg/ml. Non-specific binding on the plates was blocked with a 2% w/v solution of Bovine Serum Albumin (BSA) in PBS (blocking buffer). All sera were added to the plates at a dilution of 1 : 140 in blocking buffer. The flowthroughs and the eluents were added at a concentration corresponding to the 1 : 140 dilution of the initial IgG concentration in the human serum. After a 2-h incubation time, the plates were washed

Journal compilation © 2006 British Society for Immunology, *Clinical and Experimental Immunology*, **144:** 432–439